ORIGINAL ARTICLE

Role of tenascin C in lesion formation in early peritoneal endometriosis

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Objective: To identify cytokines or extracellular matrix components that contribute to adhesion to, and invasion of, the peritoneum, proximal to lesions in the early phase of endometriosis.
Design: Laboratory-based study.
Setting: University Hospital and Laboratory of Animal Science.
Patients and Animals: Five women with ovarian endometrioma, 138 wild-type (WT) C57BL/6N mice, and 48 *Tenascin C* (*Tnc*) knockout (TncKO) mice.
Interventions: To establish a murine endometriosis model, 20 pieces of minced uterine tissue fragments from each horn were administered intraperitoneally to syngeneic mice. Three days later, endometriotic lesions and peritoneal tissues were collected. Separately, we transfected human peritoneal mesothelial cells (HMrSV5) or human endometrial stromal cells (hESCs) with *Tnc* small interfering ribonucleic acid.
Main Outcome Measures: We employed a polymerase chain reaction array to profile gene expression in the murine peritoneum, in both peritoneum distal to lesions and peritoneum surrounding lesions (PSL). The expression of upregulated genes in the PSL was verified in the peritoneal samples by real-time reverse transcription-polymerase chain reaction. TncKO mice were used to investigate the role of Tnc in the development of endometriosis. We evaluated the proliferative activity or inflammatory state of lesions by Ki67 or CD3 immunostaining. Intraperitoneal distribution of macrophages was assessed by fluorescence-activated cell sorting. Using *Tnc* small interfering ribonucleic acid, we examined the invasive capacity of hESCs in a coculture system with HMrSV5.
Results: *Tnc* gene expression was significantly higher in PSL than in peritoneum distal to lesions. The weight and number of TncKO lesions in TncKO hosts were lower than those of WT lesions in WT hosts. In contrast, the weight and number of nonattached TncKO lesions in TncKO hosts were higher than those of nonattached WT lesions in WT hosts. We observed decreased Ki67-positive cells or H-scores for CD3, a lower proportion of M1 macrophages, and a higher proportion of M2 macrophages in TncKO lesions in TncKO recipients. Silencing of *Tnc* expression in hESCs and HMrSV5 diminished the invasivity of hESCs.
Conclusion: *Tnc* may be a crucial factor in the development of early peritoneal endometriosis. (Fertil Steril Sci® 2023;■:■–■. ©2023 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, tenascin, murine model, peritoneum, early phase

Endometriosis, defined as the presence of endometrium-like tissue outside the uterus, is a significant gynecological disease resulting in dysmenorrhea, chronic pain, dyspareunia, and infertility (1). Endometriosis is hypothesized to reflect retrograde menstruation. Although approximately 90% of females experience retrograde menstruation, only 10% would develop endometriosis. The mechanisms underlying these observations remain poorly understood. The initial stage of lesion formation is thought to require the adherence of endometrial cells to the peritoneum. The combination of the factors of inflammation, hormonal environment, and immune system is well known to exacerbate endometriosis. The early developmental stages of endometriosis are most likely related to the immune system (2). Although accumulating data have served to clarify the mechanism of endometriosis, the precise pathophysiology of early endometriosis has not been fully elucidated. We hypothesized that molecular alterations in the peritoneum are involved in developing peritoneal endometriosis.

This disease shares some characteristics with cancer, given that endometrial cells may invade adjacent organs and even spread to distant organs. Nevertheless, the mechanism of such invasion and the metastatic processes remain poorly understood. One hypothesis—the "seed and soil theory"—suggests that some tumor cells grow preferentially in the microenvironment of selected organs (3). According to this

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theory, the formation of endometriotic lesions depends on endometrial cells (seed) and peritoneal tissue (soil).

Regarding ovarian cancer, degradation and production of the extracellular matrix (ECM) are known to contribute to early events of metastasis (4). Previous literature concerning the etiology of endometriosis has shown that collagen types I and IV, tenascin C (Tnc), fibronectin, and laminin are all present in the peritoneal ECM; each of these components may serve as a potential binding site for the endometrial cells (5). The ECM elements are known to promote subsequent fibrinolysis, coagulation, and the mobilization of immune cells. Pathological accumulation of ECM is the foundation for tissue structure, facilitating interactions with the milieu and controlling cell adhesion and invasion. Furthermore, peritoneal mesothelial cells secrete numerous proinflammatory cytokines and growth factors in cell development (6, 7).

To date, several investigators have assessed the characteristics of endometriotic lesions and eutopic endometrial tissue in the pathogenesis of endometriosis. Despite the general acceptance of the retrograde implantation theory of endometriosis, the possible interaction mechanism between endometrial cells and peritoneal tissues has yet to be proven. To our knowledge, few studies have focused on the role of peritoneal tissues in the early onset of endometriosis (5, 8, 9). In the present study, we aimed to identify the crucial factor(s) expressed in the peritoneal tissues proximal to murine endometriotic lesions using a polymerase chain reaction (PCR) array of genes encoding ECM components and adhesion molecules. Notably, the peritoneum proximal to endometriotic foci exhibited strong expression of tenascin C–encoding messenger ribonucleic acids (mRNAs) (*Tnc*) during the early phase of endometriosis. Tnc may be involved in embryonic development, cancer tissues, and tissue repair at sites of inflammation. Tnc has been shown to contribute to cell differentiation, proliferation, and migration (10). On the basis of these results, we developed an in vivo murine endometriosis model using *Tnc* gene–deficient mice and an in vitro model employing coculture of human endometrial and peritoneal cells.

MATERIALS AND METHODS

Animal Care and Treatment

The animal experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Tottori University Faculty of Medicine (approval number, 20-Y-24). All experiments involving animals described in this manuscript were shown to be ethically acceptable and, where relevant, conform to appropriate national animal usage guidelines. The processes for developing the endometriosis model mice were previously established at the animal facility of the Tottori University Faculty of Medicine (11–14).

Mature female C57BL/6N mice were obtained from CLEA Japan (Tokyo, Japan) as 7-week-old animals. All mice were ovariectomized, and estradiol valerate (0.4 μg/mouse/wk; Fuji Pharma, Tokyo, Japan) in corn oil was injected subcutaneously. The donor mice (n = 30) were then euthanized by cervical dislocation, and the uteri were harvested at necropsy. Twenty pieces of minced uterine tissue fragments (<1 mm^3 each) from each horn were suspended in 300 μL of sterile saline and administered by injection into the peritoneal cavities of recipient mice (n = 60) (i.e., 1:2 ratio of the donor uterus to recipient). The recipient mice were subjected to necropsy 3 days after uterine transplantation (day 3). Specifically, we collected the normal peritoneum distal to lesions (PDL) and the peritoneum surrounding lesions (PSL), which we defined as peritoneal tissues proximal (within a 2-mm diameter) to the lesion. Sham mice received the same procedure to collect the peritoneum (PS) as experimental mice but were injected with saline alone. The tissue samples were weighed, measured, and photographed. Histologic assessment of lesions at days 1, 3, and 5 indicated that lesions at day 3 exhibited attachment, implantation, and growth representative of early-stage endometriosis (data not shown), consistent with the findings of Burns et al. (2). Therefore, day 3 was selected for subsequent evaluation of lesions. For this experiment, nonattached lesions were defined as cells that were floating in the peritoneal cavity or lesions that were flushed easily.

Construction of *Tenascin C*–Deficient Mice and Their Use in the Murine Endometriosis Models

The cryopreserved spermatozoa of the *Tnc* knockout (TncKO) mouse (B6.Cg-Tnc<tm1Sia>/Rbrc: BioResource Research Center [BRC] No. RBRC00169) were obtained from The RIKEN BRC through the National BioResource Project of MEXT/AMED (Ibaraki, Japan) (15–18). The cryopreserved spermatozoa were subjected to in vitro fertilization (IVF) with oocytes collected from C57BL/6N female mice purchased from CLEA Japan. The genotypes of the offspring from this first IVF were all heterozygous because the genotypes of the cryopreserved sperm and oocytes used for IVF were homozygous and wild-type (WT), respectively. The heterozygous males and females obtained from the first IVF were subjected to a second IVF to obtain homozygous mice. In addition, homozygous males and heterozygous females obtained in the second IVF were subjected to the third IVF to obtain more homozygous female mice. Consequently, homozygous ($Tnc^{-/-}$) females obtained from the second and third IVF were used to generate an endometriosis model (Supplemental Fig. 1, available online). The status of the *Tnc* gene in the resulting animals was determined by PCR analysis of genomic deoxyribonucleic acid (DNA), permitting the identification of *Tnc*-null mutant mice ($Tnc^{-/-}$) for use in subsequent experiments. C57BL/6N mice purchased from CLEA Japan were used as WT animals because WT mice obtained in the second IVF were not used because of the insufficient numbers for experiments. All mice (n = 48) were ovariectomized and injected subcutaneously with estradiol valerate, as described earlier. Groups were designated on the basis of the donor to recipient genotypes (respectively), as follows: WT to WT; WT to TncKO; TncKO to WT; and TncKO to TncKO. The mouse endometriosis model was generated as described previously (11–13, 19). The recipient mice were subjected to necropsy

and collection of peritoneal tissues, peritoneal fluid, and early endometriotic lesions on day 3.

PCR Array and Real-Time Reverse Transcription PCR

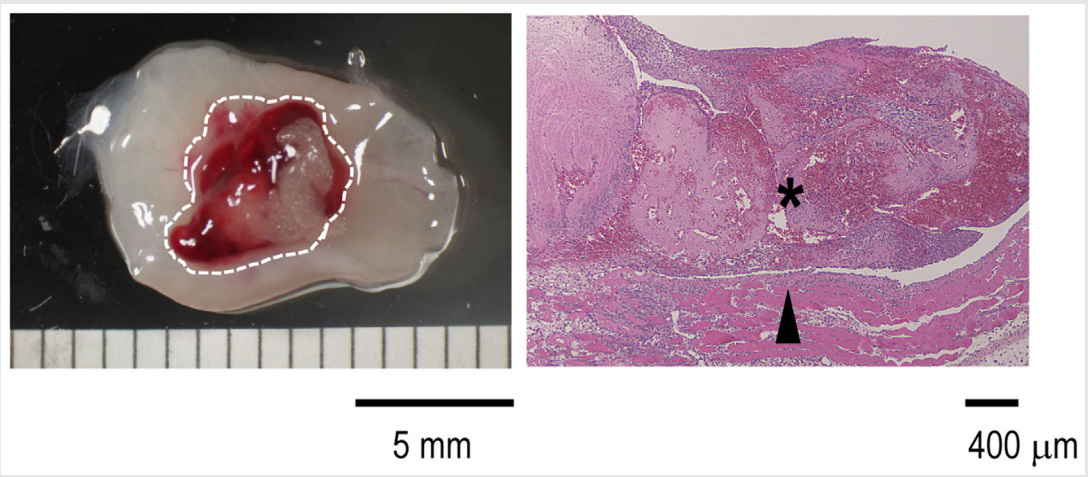
Total ribonucleic acid (RNA) was extracted from PSL and PDL samples using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan). The RNA (1 μg) from the peritoneal tissues was subjected to reverse transcription (RT) into complementary DNA (cDNA) using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan). The target mRNA levels were quantified using the ViiA 7 real-time PCR system (Applied Biosystems, Inc., Tokyo, Japan). The resulting tissue cDNA samples (PSL, n = 3; PDL, n = 3) were analyzed using the RT^2 Profiler PCR Array Mouse Extracellular Matrix and Adhesion Molecules Kit (Qiagen); this kit permits quantification of the transcript levels of 84 genes encoding transmembrane proteins and proteins known to be involved in cell-cell and cell-matrix adhesions. The fold change was calculated using Qiagen's online website analysis tools.

TaqMan probes (Applied Biosystems, Inc.) for each gene were used in the real-time RT-PCR analysis. Peritoneal samples were collected on day 3 after the transplantation of uterine tissues (PS, n = 20; PSL, n = 20; PDL, n = 20). Expression of genes encoding the following ECM and adhesion factors was evaluated: integrin α2 (*Itga2*); integrin αx (*Itgax*); matrix metallopeptidase 10 (*Mmp10*); matrix metallopeptidase 14 (*Mmp14*); selectin-endothelial type (*Sele*); selectin-platelet type (*Selp*); *Tnc*; and versican (*Vcan*). Expression was normalized to the expression of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in the respective sample. All samples were tested in triplicate, and each run included no-template and no-RT controls.

Immunohistochemical Staining

Murine endometriotic lesions and PSL were fixed and paraffin-embedded, sectioned at 5-μm thicknesses, and deparaffinized by sequential washing with xylene and a series of ethanol solutions. Immunohistochemical staining was performed as described previously (19). Staining was performed using antibodies with specificity for Ki67 (proliferation marker; Abcam, Tokyo, Japan) and CD3 (T cell marker; Abcam) as the primary antibodies. Murine spleen tissues were used as a positive control. The negative control reactions were performed by omitting the primary antibody. The Ki67- or CD3-positive and Ki67- or CD3-negative nuclei of epithelial and stromal cells were counted; the mean proportion of positive cells was calculated across three fields in a given section on each slide. A semiquantitative analysis (H-score) for CD3 staining in the epithelium and stroma of the lesions was performed, such that the cells were scored on the basis of a 4-point scale of staining intensity, as follows: 0, no staining; 1, weak; 2, medium; and 3, strong. The H-score itself was determined using the formula 1× (% of 1+ cells) + 2× (% of 2+ cells) + 3× (% of 3+ cells). The resulting scores ranged from 0 to 300. Two investigators assessed staining independently; the intraobserver and interobserver variabilities were 6% and 10% for Ki67 and 10 and 16% for CD3, respectively. For a given parameter, the mean of the scores obtained by the two investigators was used.

FIGURE 1



Representative macroscopic and microscopic views of murine peritoneal tissues and early endometriotic lesions on day 3 after transplantation of uterine tissues. The white dotted line indicates an endometriotic lesion. The peritoneal tissue proximal (within 2 mm) to the lesion was defined as the peritoneum surrounding lesions. In the hematoxylin-eosin–stained specimen, the asterisk and arrow indicate an attached lesion and the peritoneal tissue of the recipient animal, respectively.

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Flow Cytometric Analysis

To collect the peritoneal lavage, the peritoneal cavity was washed with 3 mL of phosphate-buffered saline containing 0.5% bovine serum albumin and 2-mM ethylenediaminetetraacetic acid, the abdomen was massaged gently, and the peritoneal lavage was collected. The anti-Ly-6C antibodies (labeled with fluorescein isothiocyanate) were obtained from BD Biosciences, Tokyo, Japan; the anti-F4/80 antibodies (labeled with phycoerythrin) were obtained from Invitrogen (Tokyo, Japan). The cells were diluted with 5 mL of fluorescence-activated cell sorting buffer (BD Biosciences), and antibodies were added. Stained cells were analyzed on a BD LSR Fortessa cell analyzer (BD Biosciences). Total macrophages were isolated by gating for SSC-A^{hi} and $F4/80^{+}$, and the resulting pool of total macrophages then was sorted by gating for M1 macrophages or proinflammatory macrophages ($F4/80^{med}$, $Ly6C^{-}$) and M2 macrophages or resident anti-inflammatory macrophages ($F4/80^{hi}$, $Ly6C^{-}$).

Invasion Assay in the Coculture System Using Human Endometrial and Peritoneal Cells

The experimental protocol for this project was approved by the Institutional Review Boards of the Tottori University Faculty of Medicine (approval number, 20A205), and informed consent was obtained from each patient. Endometrial tissues in the proliferative phase were collected by endometrial curettage at the time of laparoscopic surgery for endometriosis at the Tottori University Hospital. None of the patients had received hormonal therapy in the 3 months preceding surgery, and all reported having regular menstrual cycles. Human endometrial stromal cells (hESCs) were isolated from the endometrial tissues according to the previously described protocols (11, 12). The hESCs were propagated as a monolayer culture; the cells used for this study corresponded to cells obtained after the third passage. The hESCs were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin G, and 100 mg/mL of streptomycin; cells were grown at 37°C in a 5% carbon dioxide environment. The human peritoneal mesothelial cell line, HMrSV5 (20), was kindly provided by Dr. PM Ronco (Pierre and Marie Curie University, Paris, France); this line was propagated under the same conditions as those used for the hESCs.

Tnc or control small interfering RNA (siRNA) was transfected into HMrSV5 cells and hESCs using ScreenFect siRNA (Fujifilm, Osaka, Japan). Assays were conducted using Matrigel Invasion Chambers (Corning, NY). Two days after siRNA transfection, HMrSV5 cells were inoculated into the lower chamber of the system using the culture medium indicated earlier. Then, hESCs (2.0×10^4 per chamber) were seeded into the upper Matrigel Transwell insert or the insert lacking Matrigel with serum-free Dulbecco's Modified Eagle Medium and cocultured for 17 hours. After incubation, the membranes were stained with Diff-Quik (Sysmex, Hyogo, Japan), and the number of cells appearing on the undersurface of the membranes was counted in three fields at a magnification of ×200. The proportions of invasive cells (%) were calculated as the number of cells that invaded the Matrigel divided by the number of cells that migrated to the normal membranes × 100. Two investigators counted the number of cells independently and calculated the proportions of invasive cells; the intraobserver and interobserver variabilities were 5% and 9%, respectively. The mean of the scores obtained by the investigators was used.

TABLE 1

Genes with significant changes in the expression levels in the peritoneum proximal to or distal to lesions in the mouse extracellular matrix and adhesion molecule array.

Gene name	Fold change PSL/PDL on day 3
Cd44	4.40 (± 1.40)
Cdh1	0.19 (± 0.04)
Cdh2	3.60 (± 2.36)
Col2a1	0.19 (± 0.06)
Col3a1	3.03 (± 0.48)
Ctgf	3.72 (± 0.56)
Itga2	1.49 (± 0.48)
Itgae	3.19 (± 0.35)
Itgal	3.66 (± 0.71)
Itgax	9.87 (± 4.17)
Mmp10	1.58 (± 1.06)
Mmp14	2.21 (± 1.37)
Sele	4.49 (± 3.88)
Sell	4.23 (± 0.86)
Selp	1.83 (± 0.83)
Spp1	18.67 (± 6.07)
Thbs1	5.49 (± 2.20)
Timp1	15.30 (± 2.50)
Tnc	28.79 (± 15.37)
Vcan	4.13 (± 1.83)

Note: Data are presented as means (± standard errors of the mean) of 3 separate experiments. These genes encoded proteins as follows: *Cd44*, CD44; *Cdh1*, cadherin 1; *Cdh2*, cadherin 2; *Col2a1*, collagen type IIα1; *Col3a1*, collagen type IIIα1; *Ctgf*, connective tissue growth factor; *Itga2*, integrin α2; *Itgae*, integrin αE; *Itgal*, integrin αL; *Itgax*, integrin αX; *Mmp10*, matrix metallopeptidase 10; Mmp14, matrix metallopeptidase 14; *Sele*, selectin, endothelial cell; *Sell*, selectin, lymphocyte; *Selp*, selectin, platelet; *Spp1*, secreted phosphoprotein 1; *Thbs1*, thrombospondin 1; *Timp1*, tissue inhibitor of metalloproteinase 1; *Tnc*, tenascin C; and *Vcan*, versican. The "Fold change" was the ratio of the messenger ribonucleic acid expression level in the PSL compared with that in the PDL. PDL = peritoneum distal to lesions; PSL = peritoneum surrounding lesions.

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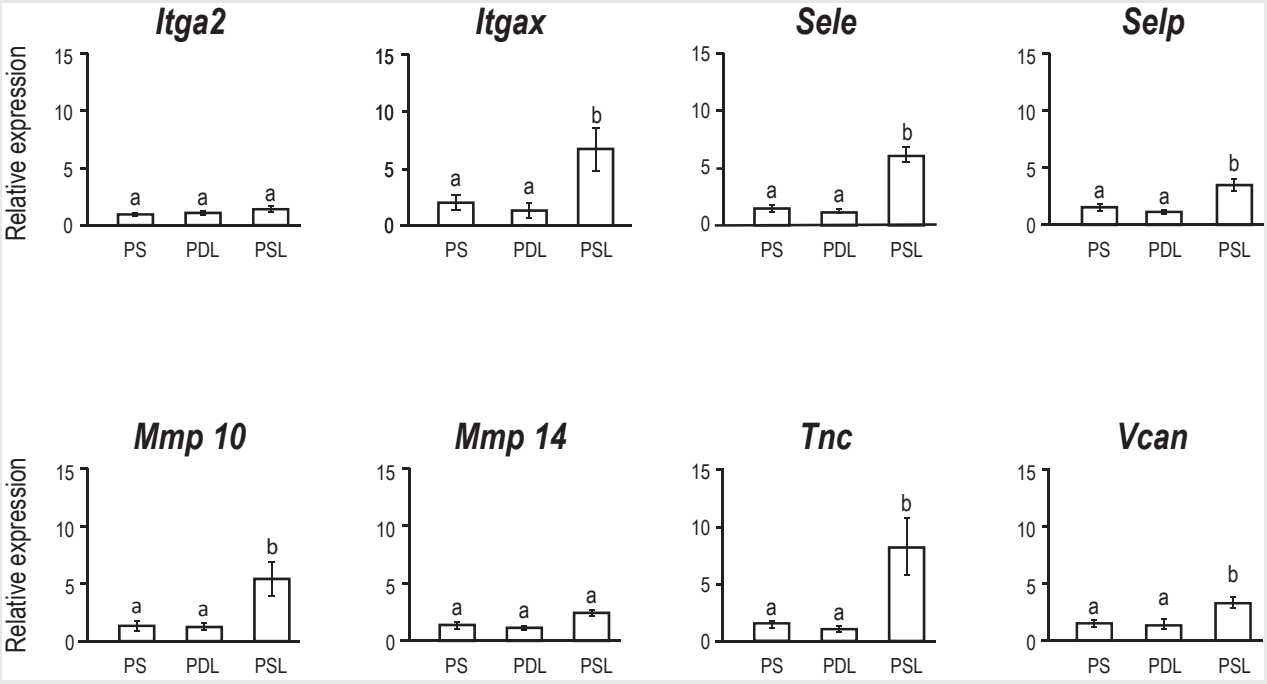
Statistical Analysis

After confirming normality and equal variance using the Kolmogorov-Smirnov and Bartlett tests, multiple comparisons were performed using one-way analysis of variance with the post hoc Tukey tests (for parametric data) or using the Kruskal-Wallis test with the post hoc Steel-Dwass tests (for nonparametric data). All comparisons were run as two-tailed tests. A P value of $<.05$ was considered statistically significant. Data are presented as the means and SEMs. All statistical analyses were performed using the EZR software package (version 1.61; Jichi Medical University Saitama Medical Center, Saitama, Japan).

FIGURE 2



Real-time reverse transcription-polymerase chain reaction was used to examine the expression of mouse genes encoding extracellular matrix and adhesion-associated proteins. We collected the samples on day 3 after the transplantation of uterine tissues. Data are presented as means ± SEMs (n = 60). All experiments were repeated a minimum of three times. The mean not sharing a letter significantly differed ($P<.05$). PDL = peritoneum distal to lesions; PS = sham peritoneum, peritoneum without injection of uterine fragments; PSL = peritoneum surrounding lesions.

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RESULTS

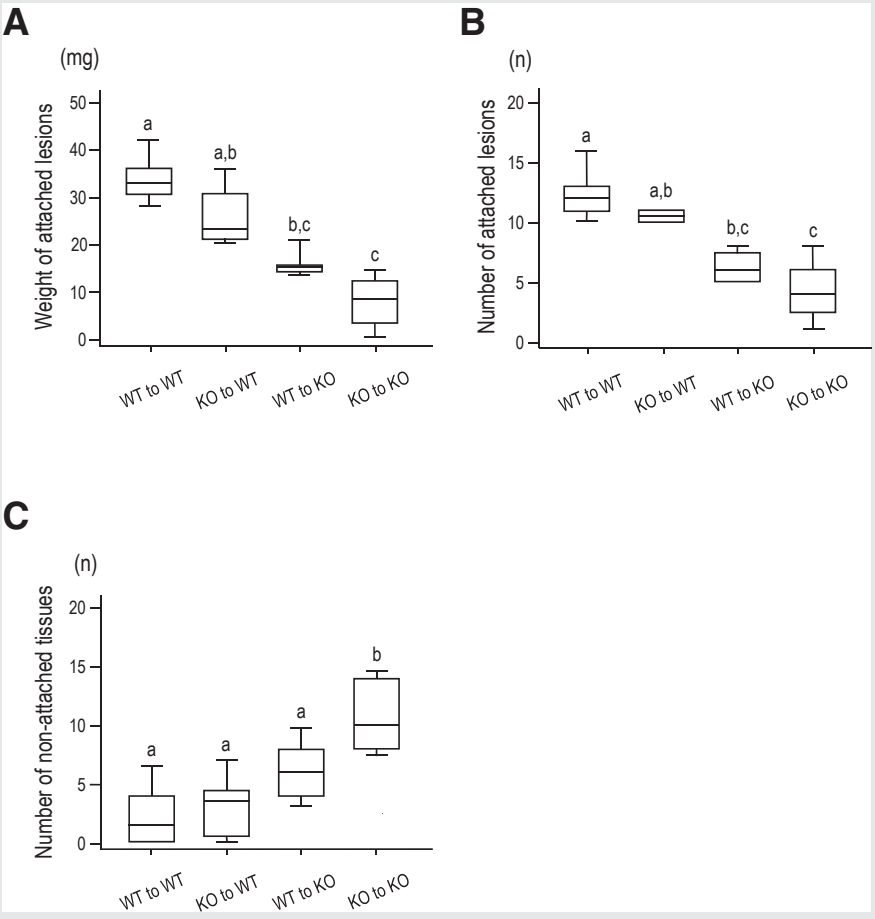
Expression of the ECM- and Adhesion Molecule-Encoding Genes in the Murine Peritoneum

On day 3 after initiation of disease by tissue implantation in mice, we observed peritoneal lesions that exhibited macroscopic and microscopic characteristics consistent with murine endometriosis (Fig. 1). To elucidate the molecular basis whereby early-phase endometriotic tissues became connected to the peritoneum, gene expression profiling of the day 3 peritoneal tissues was performed using a PCR array to detect transcripts encoding ECM and adhesion molecules. For this analysis, the cDNAs isolated from the peritoneal tissues of four mice were pooled into a single sample. An increased expression level was defined using a cutoff value of 1.5-fold change in PSL or PDL. Several genes, such as *Tnc*, versican (*Vcan*), selectin-platelet type (*Selp*), integrin αX (*Itgax*), and secreted phosphoprotein (*Spp1*) demonstrated increased expression levels in PSL compared with those in PDL; in contrast, the levels of transcripts encoding cadherin 1 (*Cdh1*) and collagen type II alpha1 (*Col2a1*) were lower in PSL than in PDL. The levels of the transcripts encoding *Tnc*, *Vcan*, and *Selp* expression differed between PSL ($C_t < 30$) and PDL ($C_t > 30$) (Table 1). Next, we assessed the expression of selected genes using quantitative RT-PCR. Specifically, we analyzed the *Itgax*, selectin-endothelial cell type (*Sele*), *Selp*, matrix metallopeptidase 10 (*Mmp10*), *Tnc*, and *Vcan* transcripts as representative highly expressed genes identified via the PCR array. The RT-PCR analysis verified that these transcripts accumulated to higher levels in PSL than in PDL on day 3. The *Tnc* expression in PSL increased significantly (approximately 7.5-fold compared with that in PS or PDL) among these genes (Fig. 2).

Evaluation of Endometriotic Lesions Using *Tenascin C*–Deficient Mice

To address the role of Tnc in endometriosis, we established a syngeneic murine endometriosis model in *Tnc* gene–deficient mice. We used WT and TncKO mice in four donor-recipient combinations: WT-WT; WT-TncKO; TncKO-WT; and TncKO-TncKO. For all combinations, endometriotic lesions were observed in the peritoneal cavity, where the donor tissues were observed to adhere primarily to the peritoneal wall and to fat tissues, and adjacent to the bladder. We next assessed the weight and number of endometriotic lesions in each mouse. The attached lesions in TncKO-TncKO were statistically significantly ($P<.05$) lower in weight and fewer in number than those in WT-WT (weight, 8.0 vs. 33.2 mg, respectively; number, 4.0 vs. 12.0) (Fig. 3A and B). In the comparison of WT-WT with WT-TncKO, as well as that of TncKO-WT with TncKO-TncKO, the weights and numbers of the

FIGURE 3



Comparison of murine endometriotic lesions in the four groups on day 3. The assessed parameters included the total lesion weight (A), number of attached lesions (B), and number of nonattached lesions (C). Wild-type (WT) and *Tenascin C* (*Tnc*) knockout (KO; TncKO) mice were used as donors or recipients, with groups annotated in the form of A to B (donor to recipient). Data are presented as means ± SEMs (each group, n = 16). The means not sharing a letter significantly differed ($P<.05$).

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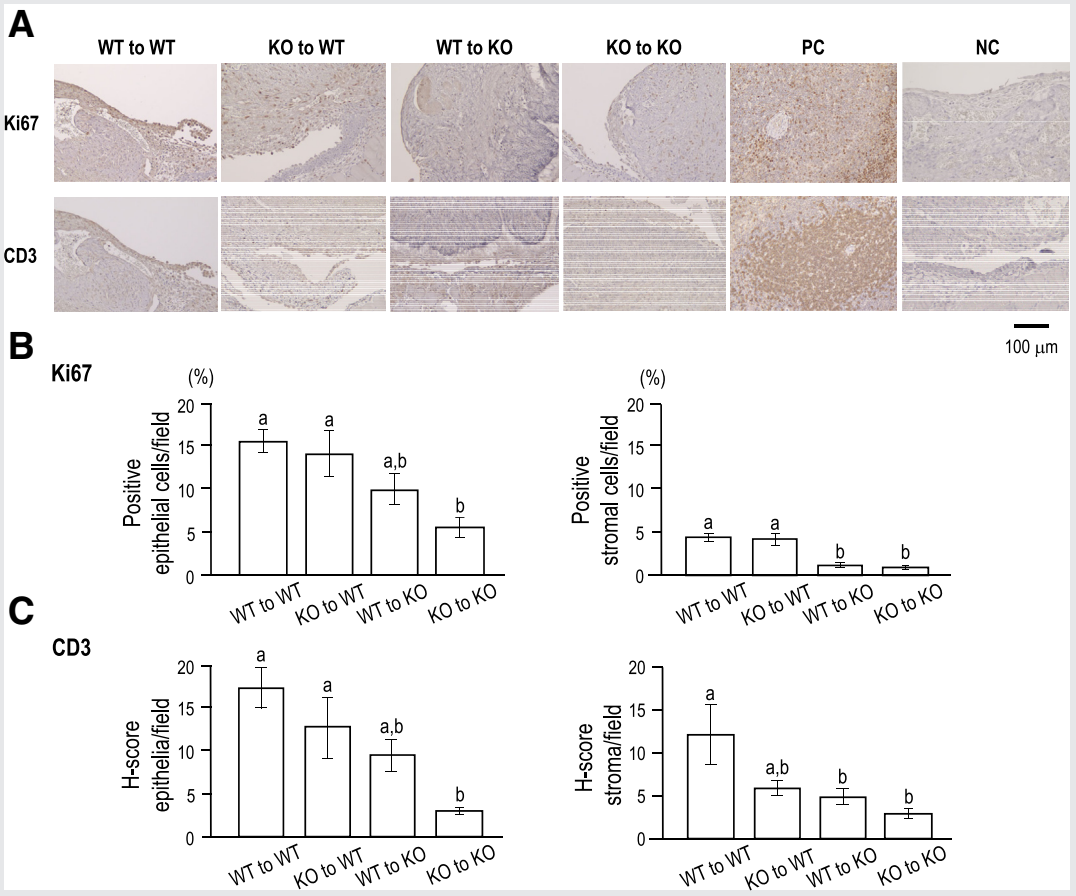
attached lesions were significantly smaller in the second member of each comparison (WT-TncKO and TncKO-TncKO) than in the respective comparator ($P<.05$) (Fig. 3A and B), suggesting that Tnc expression in the peritoneum is crucial to the attachment of endometrial foci to the peritoneum. In contrast, a statistically significantly ($P<.05$) larger number of nonattached lesions (observed floating with the peritoneal cavity) were detected in TncKO-TncKO than in WT-WT (10.0 vs. 1.5, respectively) (Fig. 3C).

We next investigated potential differences between the four donor-recipient combinations in proliferative activity and inflammatory reaction. Specifically, we assessed proliferation and T cell aggregation by immunofluorescent staining for Ki67 or CD3 antigens (Fig. 4A). The positive rates of Ki67 staining, as well as the H-scores for CD3 staining, in epithelial and stromal cells of endometriotic lesions in TncKO-TncKO were significantly smaller than those in WT-WT. For Ki67, the staining value (mean ± SEMs) in epithelial tissue was 17.1% ± 1.4% in WT-WT vs. 5.5% ± 1.2% in TncKO-TncKO ($P<.05$), whereas that in stromal tissue was 4.3% ± 0.4% vs. 1.0% ± 0.1% ($P<.05$), respectively. For CD3, the staining value in epithelial tissue was 17.3% ± 2.4% in WT-WT vs. 9.3% ± 2.2% in WT-TncKO ($P=.11$) and vs. 3.0% ± 0.4% in TncKO-TncKO ($P<.05$), whereas that in stromal tissue was 12.3% ± 0.4% in WT-WT vs. 5.0% ± 1.0% in WT-TncKO ($P<.05$) and vs. 3.0% ± 0.6 in TncKO-TncKO ($P<.05$) (Fig. 4B and C).

Distribution of Intraperitoneal Macrophages in *Tenascin C*–Deficient Mice

We examined differences in the distribution of macrophages in the peritoneal lavage of the various implantation groups. The expression markers F4/80 and Ly6C were used to distinguish M1 and M2 macrophages (Fig. 5A). When TncKO mice were used as donors or recipients, the proportion of M1

FIGURE 4



Immunohistochemical analysis of Ki67 and CD3 expression in early endometriotic lesions in the four groups. (A) Tissues stained with antibodies against Ki67 or CD3. (B) The proportions of Ki67-positive cells and (C) H-scores for CD3 staining in the epithelia and stroma of the lesions. Data are presented as means ± SEMs (each group, n = 6). The means not sharing a letter significantly differed ($P<.05$). NC = negative control; PC = positive control; KO = knockout; WT = wild-type.

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macrophages decreased, and that of M2 macrophages increased. Specifically, the proportion of M1 cells was 58.3% ± 3.65% in WT-WT vs. 37.5% ± 3.35% in TncKO-TncKO ($P<.05$). Furthermore, the proportion of M2 cells was 29.8% ± 3.10% in WT-WT vs. 49.5% ± 3.78% in TncKO-TncKO ($P<.05$) (Fig. 5B).

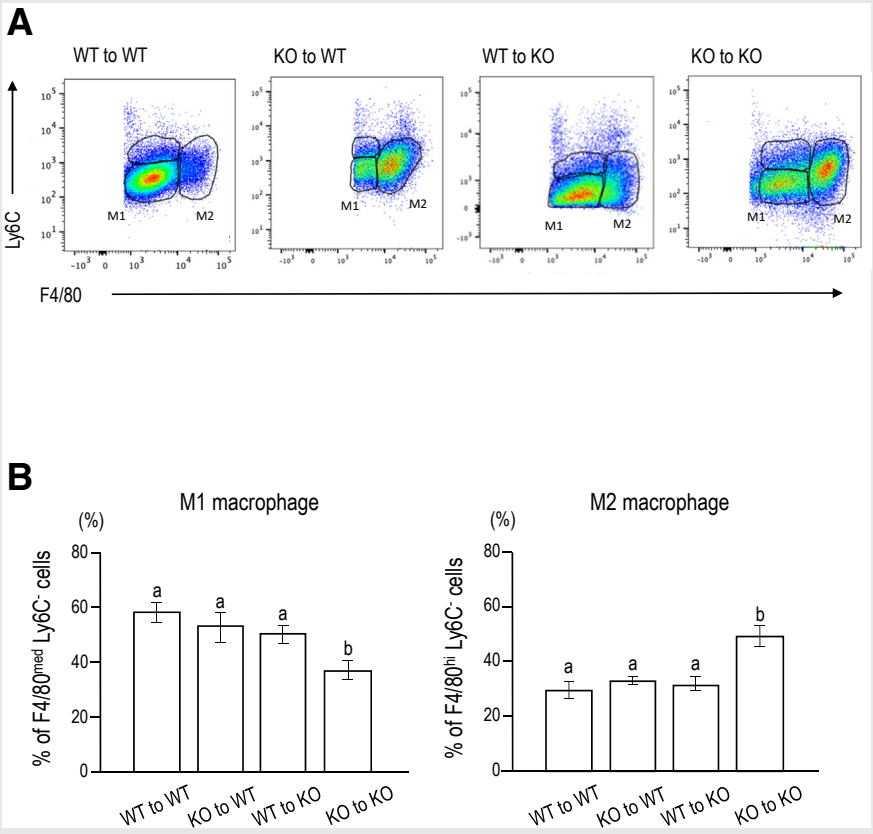
Silencing of Tenascin Expression Has a Negative Impact on Invasivity

Next, we performed coculture experiments using Matrigel chambers to assess the effect of *Tnc* deficiency on the invasivity of hESCs. First, we confirmed that transfection of Tnc-siRNA into HMrSV5 or hESCs reduced the level of *Tnc* mRNA by >90% (data not shown). Compared with control cocultures (transfected with the control siRNA), cocultures in which *Tnc* expression was silenced in both HMrSV5 and hESCs exhibited a statistically significant ($P<.05$) decrease of approximately 15% in the proportion of invasive hESCs (102% ± 2.3% vs. 84% ± 5.3%, respectively) (Fig. 6).

DISCUSSION

Endometriosis is challenging to treat. The disease resembles a malignant disease, given the involvement of cell migration, invasion, and proliferation. Although researchers have attempted to understand the origin of this disease and its pathological causes, the etiology of endometriosis remains controversial. The main goal of the present study was to identify ECM and adhesion molecules that are essential to the onset of endometriosis. When the refluxed endometrium initially binds to the pelvic cavity, the tissue attaches to the ECM, leading to the expression of *Tnc* mRNA in the peritoneal base membrane. Our examination of the connection between the endometrial fragments and peritoneum was expected to clarify the mechanism of endometriotic lesion formation.

FIGURE 5



Distributions of M1 and M2 macrophages in the peritoneal lavage of recipient mice. (A) Macrophage gating strategy. Total macrophages were isolated by gating for SSC-A^{hi} and F4/80^{+}; the resulting cells then were gated for F4/80^{+} and Ly6C^{-} to identify M1 and M2 macrophages, respectively. (B) Proportions of M1 and M2 macrophages (among the total macrophages). Data are presented as means ± SEMs (n = 48). The means not sharing a letter significantly differed ($P<.05$). KO = knockout; WT = wild-type.

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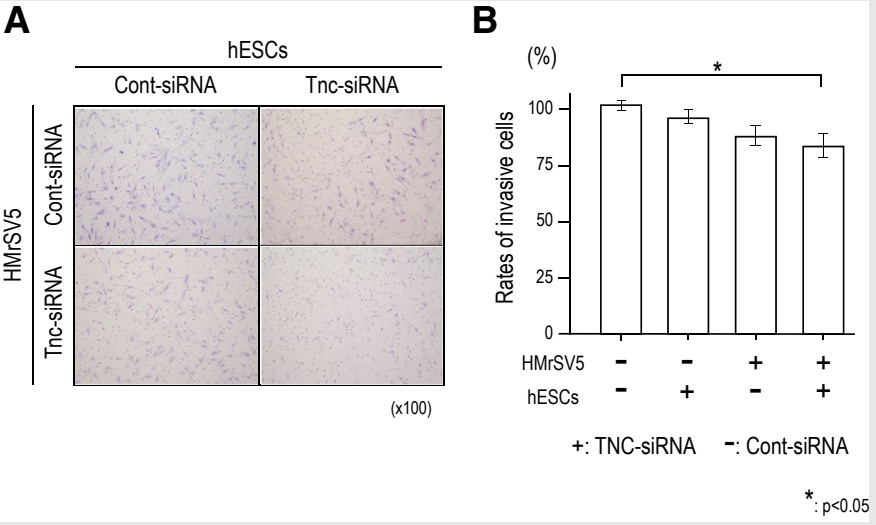
Our data showed that the *Tnc* transcript accumulates in the murine peritoneum proximal to lesions during the early stages of endometriosis; the use of *Tnc*-deficient recipient mice resulted in attenuation of the attachment of ectopic uterine tissue to the peritoneum. In addition, we assessed the intraperitoneal environment in our mouse model of endometriosis using flow cytometric analysis to examine the distribution of macrophages in the peritoneal lavage (Fig. 5). We confirmed these results by showing that silencing of *Tnc* in a human peritoneal cell line (HMrSV5) and hESCs decreased the invasivity of hESCs in an in vitro coculture model. On the basis of these data, we infer that Tnc plays a crucial role in regulating attachment and invasion by hESCs.

Because endometriosis is observed only in humans and a subset of primates (e.g., monkeys) and a limited number of experimental models are available, research on early-stage endometriotic lesions has remained challenging to perform. Given the expense of conducting endometriosis research in primates, murine models have been developed to investigate the pathophysiology and efficacy of treatments for this disease. Endometriotic lesions do not occur spontaneously in mice, presumably reflecting their closed reproductive organs. However, we have successfully created a murine endometriosis model using the transplantation of donor uterine tissue into the peritoneum of recipient mice (11–14, 19). Although several experiments have been performed using endometriotic lesions, to our knowledge, no previous study has focused on the difference between the peritoneum proximal to the lesion and the normal peritoneum. For our coculture experiments, we chose to use hESCs to assess cell invasion; this decision was based on the work of Witz et al. (21), who verified that hESCs attach and invade the mesothelial surface of the peritoneum, subsequently growing into endometriotic lesions. This and other studies demonstrate that the peritoneum plays a vital role in the initial step of lesion formation.

In general, Tnc is expressed in the nervous, muscular, and vascular systems during embryonic development; in adults, this protein is expressed in cancer tissues and during tissue repair at sites of inflammation. Tnc is a large, hexameric ECM glycoprotein and has been shown to contribute to cell differentiation, proliferation, and migration (10). Tnc may also

FIGURE 6



Effect of *Tenascin C* gene silencing on the invasivity of human endometrial stromal cells (hESCs) cocultured with human peritoneal mesothelial cells (HMrSV5). (A) Representative photos. (B) Rates of hESC invasivity. Data are presented as means ± SEMs (n = 5). Comparisons were performed using two-tailed one-way analysis of variance with the post hoc Tukey tests. *$P<.05$. Cont-siRNA = control small interfering ribonucleic acid; Tnc-siRNA = *tenascin C* small interfering ribonucleic acid.

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serve as a soluble factor, not only in peritoneal tissue but also in blood vessels and ascites fluid (22–24). We have observed intense Tnc expression in the stroma of the murine uterus (25). In humans, an increased tenascin expression level is observed in the stroma of the functionalis of the endometrium during the proliferative phase (26). Another laboratory reported that Tnc is expressed at higher levels in the proliferative endometrium than in the secretory endometrium; the most potent expression is detected in endometriotic implants (27). Those investigators also showed that exposure to exogenous estradiol resulted in the accumulation of Tnc protein in hESCs (27).

We used a PCR array to analyze the expression, in the peritoneal tissues surrounding the lesions, of genes encoding other ECM factors (Table 1 and Fig. 2). We observed changes in *Vcan* expression, consistent with a previous study showing that *Vcan* is more strongly expressed in the peritoneal tissues derived from women with endometriosis than in women without endometriosis, and hyaluronan and CD44-mediated versican-induced attachment of hESCs to the peritoneum (8). We also observed changes in the expression of the genes encoding several integrins, a class of proteins implicated in the pathogenesis of endometriosis (28, 29). For instance, integrin alpha X acts as a fibrinogen receptor and mediates cell-cell interaction during inflammatory responses (e.g., macrophage attachment). In another study, Guo et al. (30) demonstrated that a deficiency of *Selp* impeded the development of endometriosis in a murine model; those investigators suggested that targeting of *Selp*-mediated adhesion of platelets to endometriotic lesions is of use in treating endometriosis.

In our coculture system, *Tnc* gene silencing in both HMrSV5 and hESCs resulted in decreased invasivity of hESCs (Fig. 6), suggesting that Tnc facilitates attachment and invasion in the early phase of endometriosis. Notably, this experiment showed that Tnc should perform this function by acting as a soluble factor, given the use of growth chambers that separate physically the cocultured cells. These results imply that Tnc controls inflammatory status during the early stages of the formation of endometriotic lesions.

This study has several limitations. First, our analysis was confined to gene expression, histology, and immunohistochemistry and lacked any molecular tests of the mechanism(s) associated with Tnc's function. In addition, we did not determine the localization of Tnc protein expression in the mouse tissues. However, *Tnc* expression was evident in the peritoneum and early-stage endometriotic lesions. TncKO mice remain viable and can conceive without any apparent anomalies. Existing medical therapies for endometriosis aim to decrease ovarian estrogen production and/or counteract the effects of estrogen. Tnc may be an effective therapeutic target for endometriosis patients who wish to avoid hormonal inhibition or who still wish to conceive. Inhibition of Tnc expression or function may prevent the formation or progression of initial endometriotic lesions in adolescents or young women or the recurrence of this disease.

CONCLUSION

In conclusion, our findings implicate Tnc in the interaction between the peritoneum and endometriotic lesions in the early stages of endometriosis. Further studies will be needed to elucidate the role of Tnc in cell adhesion and invasion in the development of endometriotic lesions. Our findings are expected to contribute to the identification

of diagnostic markers and/or therapeutic agents for endometriosis.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Maako Moriyama: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Kazuomi Nakamura:** Resources, Methodology, Investigation, Formal analysis. **Hiroki Nagata:** Formal analysis, Data curation. **Ikumi Wada:** Methodology, Data curation. **Kei Nagira:** Validation, Formal analysis. **Yukihiro Azuma:** Methodology, Data curation. **Eri Sato:** Methodology, Data curation. **Tasuku Harada:** Supervision. **Fuminori Taniguchi:** Writing – review & editing, Visualization, Validation, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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F&S SCIENCE CLINICAL QUICK TAKE

Tenascin C contributes to the progression of endometriotic lesions in the early stages of endometriosis; the analysis of the expression of this protein may facilitate the identification of diagnostic markers and therapeutic agents for endometriosis. Inhibition of tenascin C in the peritoneum and endometriotic lesion may be effective for patients without hormonal inhibition, notably for patients with endometriosis who still wish to conceive. Tenascin C could be a crucial target for endometriosis in adolescents or young women or to prevent the recurrence of this disease.

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